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A hierarchical analysis of the interactive effects of elevated CO$_2$ and water availability on the nitrogen and transpiration productivities of velvet mesquite seedlings

Abstract In this study we apply new extensions of classical growth analysis to assess the interactive effects of elevated CO$_2$ and differences in water availability on the leaf-nitrogen and transpiration productivities of velvet mesquite (*Prosopis velutina* Woot.) seedlings. The models relate transpiration productivity (biomass gained per mass of water transpired per day) and leaf-nitrogen productivity (biomass gain per unit leaf N per day) to whole-plant relative growth rate (RGR) and to each other, allowing a comprehensive hierarchical analysis of how physiological and morphological responses to the treatments interact with each other to affect plant growth. Elevated CO$_2$ led to highly significant increases in N and transpiration productivities but reduced leaf N per unit leaf area and transpiration per unit leaf area, resulting in no net effect of CO$_2$ on the RGR of seedlings. In contrast, higher water availability led to an increase in leaf-tissue thickness or density without affecting leaf N concentration, resulting in a higher leaf N per unit leaf area and consequently a higher assimilatory capacity per unit leaf area. The net effect was a marginal increase in seedling RGR. Perhaps most important from an ecological perspective was a 41% reduction in whole-plant water use due to elevated CO$_2$. These results demonstrate that even in the absence of CO$_2$ effects on integrative measures of plant growth such as RGR, highly significant effects may be observed at the physiological and morphological level that effectively cancel each other out. The quantitative framework presented here enables some of these tradeoffs to be identified and related directly to each other and to plant growth.

Keywords Climate change · Growth analysis · MANOVA · *Prosopis velutina* · Water use

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Introduction

In a recent review paper, Wullschleger et al. (2002) concluded that our understanding of how elevated CO$_2$ modifies plant water relations and the subsequent effects on plant growth are limited by several conceptual and experimental-design issues. Two of these issues are (1) the lack of a quantitative framework for integrating treatment effects on important physiological and morphological traits across different levels of biological organization; and (2) the difficulty in identifying the true effect of CO$_2$ versus the effect of soil-water conservation caused by reduced stomatal conductance at elevated CO$_2$. Confounding the issue further is the fact that the water and carbon economies of a plant are tightly linked to its nitrogen economy through photosynthesis. Water is lost as a result of CO$_2$ uptake and N is an essential element in the proteins involved in carbon fixation. Because of this coupling between C gain, water loss, and leaf N, it can be argued that a quantitative understanding of how elevated CO$_2$ affects plant water relations and growth requires the role of leaf N to be included.

In this paper, we present some simple extensions of classical growth analysis that unify the established concepts of leaf-nitrogen productivity and transpiration productivity with each other and with leaf and plant growth. Some of these equations differ only subtly from previously published versions, but these small differences are important because they facilitate the unification of these concepts. Because this framework is phenomenological it does not fully satisfy the need for a mechanistic framework argued for by Wullschleger et al. (2002). Nevertheless, it is particularly useful for quantifying some

of the complicated interactions that occur between transpiration, leaf N, and biomass gain and allocation. We applied this framework to an experiment on velvet mesquite (*Prosopis velutina* Woot.) seedlings subjected to two CO_2 partial pressures and two levels of water availability. To address the important issue of confounding the direct effects of elevated CO_2 with potential effects of soil-water conservation that might result from reduced stomatal conductance (De Luis et al. 1999; Wullschleger et al. 2002), we used a self-regulating subsoil watering system that maintained a constant soil-water content in all treatments.

Mesquites and other woody species in the semiarid southwestern United States have shown substantial increases in population density and geographic range since Anglo-American settlement of this region approximately 120 years ago (Van Auken and Bush 1990; Gibbens et al. 1992; Bahre and Shelton 1993; Archer 1995; Boutton et al. 1999; Van Auken 2000; Ansley et al. 2001; Wilson et al. 2001; Biggs et al. 2002). These woody species are C_3 whereas most of the native grasses they are displacing are C_4. Because many C_3 plants demonstrate higher growth rates when exposed to elevated levels of atmospheric CO_2 through reductions in substrate limitation and photorespiration (Field et al. 1992), anthropogenic increases in atmospheric CO_2 could be a factor in the historical increases in mesquite population density and/or a driving factor in their future expansion (Johnson et al. 1993). The goal of this study was to elucidate the direct effect of elevated CO_2 and differences in water availability on the leaf-N and transpiration productivities of *P. velutina* seedlings, and to quantify how these responses interacted

with each other and important morphological characteristics of the seedlings to influence whole-plant growth.

Materials and methods

Growth analysis

Following Watson (1952) and Radford (1967), the relative growth rate (RGR) of a plant can be partitioned into the product of the unit leaf rate (ULR) and leaf area ratio (LAR, see Table 1 for a list of abbreviations and units)

$$\frac{1}{W}\frac{dW}{dt} = \frac{1}{S_L}\frac{dW}{dt} \times \frac{S_L}{W}. \tag{1}$$

W is the total dry mass of the plant and S_L is the total leaf area. The LAR can in turn be partitioned into the product of specific leaf area (SLA) and the leaf mass ratio (LMR)

$$\frac{S_L}{W} = \frac{S_L}{W_L} \times \frac{W_L}{W}, \tag{2}$$

where W_L is the total leaf mass. Thus, RGR = ULR × SLA × LMR.

Hirose (1984) introduced the concept of leaf nitrogen productivity (N_{prod}, the rate of dry mass production per unit mass of N in the leaves), which Wright and Westoby (2000) related to the ULR by multiplying by leaf N per unit leaf area (N_{area}) to give

Table 1 Abbreviations, units, and time-averaged equations for the growth analysis variables. Integration of the time-averaged equations assumed that the relationships between W and W_L, W_R, W_S, S_L, S_R, N_L, and k can be approximated by linear functions, and that all variables were continuous functions of time

Abbreviation	Name	Units	Time-averaged equation
CRGR	Component relative growth rate	g g^{-1} day^{-1}	$\overline{CRGR}_i = [(W_{i2} - W_{i1})(\ln W_2 - \ln W_1)]/[(W_2 - W_1)(t_2 - t_1)]$
E	Transpiration	g cm^{-2}	$\overline{E} = [(k_2 - k_1)(\ln S_{L2} - \ln S_{L1})]/[(S_{L2} - S_{L1})(\ln k_2 - \ln k_1)]$
E_{prod}	Transpiration productivity	g g^{-1} day^{-1}	$\overline{E_{prod}} = [(W_2 - W_1)(\ln k_2 - \ln k_1)]/[(k_2 - k_1)(t_2 - t_1)]$
LAR	Leaf area ratio	cm^2 g^{-1}	$\overline{LAR} = [(S_{L2} - S_{L1})(\ln W_2 - \ln W_1)]/[(W_2 - W_1)(\ln S_{L1} - \ln S_{L1})]$
LMR	Leaf mass ratio	Dimensionless	$\overline{LMR} = [(W_{L2} - W_{L1})(\ln W_2 - \ln W_1)]/[(W_2 - W_1)(\ln W_{L2} - \ln W_{L1})]$
N_{area}	N per unit leaf area	g cm^{-2}	$\overline{N_{area}} = [(N_{L2} - N_{L1})(\ln S_{L2} - \ln S_{L1})]/[(S_{L2} - S_{L1})(\ln N_{L2} - \ln N_{L1})]$
N_L	Mass of N in leaves	g	
N_{prod}	N productivity	g g^{-1} day^{-1}	$\overline{N_{prod}} = [(W_2 - W_1)(\ln N_{L2} - \ln N_{L1})]/[(N_{L2} - N_{L1})(t_2 - t_1)]$
RGR	Relative growth rate	g g^{-1} day^{-1}	$\overline{RGR} = (\ln W_2 - \ln W_1)/(t_2 - t_1)$
RGR_{LA}	Leaf-area relative growth rate	cm^2 cm^{-2} day^{-1}	$\overline{RGR_{LA}} = (\ln S_{L2} - \ln S_{L1})/(t_2 - t_1)$
S_L	Leaf area	cm^2	
SLA	Specific leaf area	cm^2 g^{-1}	$\overline{SLA} = [(S_{L2} - S_{L1})(\ln W_{L2} - \ln W_{L1})]/[(W_{L2} - W_{L1})(\ln S_{L2} - \ln S_{L1})]$
S_R	Root area	cm^2	
S_R: S_L	Root area to leaf area ratio	Dimensionless	$\overline{S_R : S_L} = [(S_{R2} - S_{R1})(\ln S_{L2} - \ln S_{L1})]/[(S_{L2} - S_{L1})(\ln S_{R2} - \ln S_{R1})]$
ULR	Unit leaf rate	g cm^{-2} day^{-1}	$\overline{ULR} = [(W_2 - W_1)(\ln S_{L2} - \ln S_{L1})]/[(S_{L2} - S_{L1})(t_2 - t_1)]$
WURA	Water uptake per unit root area	g cm^{-2}	$\overline{WURA} = [(k_2 - k_1)(\ln S_{R2} - \ln S_{R1})]/[(S_{R2} - S_{R1})(\ln k_2 - \ln k_1)]$
ΔLMA	Change in leaf mass relative to leaf area	g cm^{-2}	$dW_L/dS_L = (W_{L2} - W_{L1})/(S_{L2} - S_{L1})$

$$\frac{1}{S_L}\frac{dW}{dt}=\frac{1}{N_L}\frac{dW}{dt}\times\frac{N_L}{S_L}, \tag{3}$$

where N_L is the total mass of N in the leaves. Combining Eqs. 2 and 3 gives

$$\frac{1}{W}\frac{dW}{dt}=\frac{1}{N_L}\frac{dW}{dt}\times\frac{N_L}{S_L}\times\frac{S_L}{W_L}\times\frac{W_L}{W}, \tag{4}$$

or RGR = $N_{prod}\times N_{area}\times$ SLA × LMR.

Using similar logic, we partition the ULR into measures of transpiration productivity (E_{prod}, the rate of dry mass production per unit mass of water transpired) and the mass of water transpired per unit leaf area (E)

$$\frac{1}{S_L}\frac{dW}{dt}=\frac{1}{k}\frac{dW}{dt}\times\frac{k}{S_L}. \tag{5}$$

Here k is the total mass of water transpired by the plant. Combining Eqs. 2 and 5 gives

$$\frac{1}{W}\frac{dW}{dt}=\frac{1}{k}\frac{dW}{dt}\times\frac{k}{S_L}\times\frac{S_L}{W_L}\times\frac{W_L}{W} \tag{6}$$

or RGR = $E_{prod}\times E\times$ SLA × LMR.

We now partition the ULR into the product of E_{prod}, water uptake per unit root area (WURA), and the root area (S_R) to leaf area ratio ($S_R:S_L$):

$$\frac{1}{S_L}\frac{dW}{dt}=\frac{1}{k}\frac{dW}{dt}\times\frac{k}{S_R}\times\frac{S_R}{S_L}. \tag{7}$$

This assumes that the mass of water stored in a plant is small relative to the mass of water transpired such that the mass of water taken up by the roots (k_R) will approximate the mass transpired by leaves (k). A similar approach can be used to relate N uptake per unit root area to the plant N content, the ULR, N_{area}, and $S_R:S_L$, but this extension of growth analysis was not used here because the species of interest is a legume and it is difficult to accurately assign the amount of plant N derived from N fixation or root uptake.

We now relate N_{prod} to E_{prod} through the ratio E/N_{area} such that

$$\frac{1}{N_L}\frac{dW}{dt}=\frac{1}{k}\frac{dW}{dt}\times\frac{k}{N_L}, \tag{8}$$

where the ratio k/N_L is the result of dividing E by N_{area}. Polley et al. (1999a) also link the nitrogen and water economies of plants by using the relationship E = plant N × (E/plant N). They claim that this relationship demonstrates that changes in plant N acquisition or changes in the relative amounts of water and N used in production will indirectly alter total transpiration. However, in their relationship plant N cancels out and therefore it is independent of transpiration. Thus, their claims that changes in plant N will indirectly affect E cannot be justified on the grounds of their mathematical relationship alone. Although our Eq. 8 could be considered mathematically similar to the relationship of Polley et al. (1999a), we recognize the mathematical independence of k and N_L. Our primary use of Eq. 8 is as a tool for explaining differences in the response of the nitrogen and water economies of plants.

Following Hunt and Bazzaz (1980), the RGR can be partitioned into components representing the additive contributions of the roots, stems, and leaves:

$$\frac{1}{W}\frac{dW}{dt}=\frac{1}{W}\frac{dW_R}{dt}+\frac{1}{W}\frac{dW_S}{dt}+\frac{1}{W}\frac{dW_L}{dt}. \tag{9}$$

The subscripts R, S, and L refer to the roots, stems and leaves, and $W_R+W_S+W_L=W$. The components of the sum in Eq. 9 are referred to as the root, stem, and leaf component relative growth rates (CRGR) respectively.

Finally, we show that the leaf CRGR can be partitioned into the product of the leaf-area relative growth rate (RGR_{LA}), the LAR, and the rate of change in leaf mass relative to leaf area (ΔLMA):

$$\frac{1}{W}\frac{dW_L}{dt}=\frac{1}{S_L}\frac{dS_L}{dt}\times\frac{S_L}{W}\times\frac{dW_L}{dS_L}. \tag{10}$$

This equation shows to what extent leaf growth is determined by changes in leaf area and/or changes in leaf tissue thickness or density. This equation can also be modified for root growth, but this was not done in this study.

Numerical values for the growth analysis variables were estimated from the data after integrating the models to give time-averaged approximations. These approximations and their associated assumptions are presented in Table 1, along with a complete list of abbreviations and their corresponding names and units.

Watering system

A self-regulating subsoil watering system based on the Snow and Tingey (1985) system, modified by Wookey et al. (1991) to incorporate a 'flask of Mariotte' (Fig. 1), was used to control the water content of the potting medium. These authors provide detailed explanations of how the system works. Plants were grown in 10.0 cm diameter by 20.0 cm high PVC tubes (pots) covered on the bottom with 25.0 μm nylon mesh (Nitex mesh, Sefar America, Kansas City, Mo.). The mesh allowed water to pass through but was too fine for roots to penetrate. The use of shallow pots can be criticized for restricting root growth and sink strength, but in the Sonoran uplands north of Tucson, mesquite is the dominant woody species in areas

where an impermeable calcic horizon exists in the soil at depths less than 30 cm. In these areas, mesquites resemble shrubby bushes, possibly in part because the shallow calcic horizon restricts vertical root growth.

Each tube was placed on a separate stack of hygroscopic foam cylinders (Aqua Foam#106, Smithers Oasis, Kent, Ohio, USA) contained inside a large PVC tube sealed at one end. Each container was connected to a modified 1,000 ml graduated cylinder using a vinyl hose. The graduated cylinder was set up as a 'flask of Mariotte' constant head device (Wookey et al. 1991) by using an airtight stopper with a glass tube inserted through its center to seal the cylinder after it had been filled with water. Atmospheric pressure regulated the height of water in the cylinder containing the foam blocks at the same level as the bottom of the glass tube in the graduated cylinder. The foam blocks conducted water to the bottom of the pot where it passed through the nylon mesh and into the potting soil. The system maintained a constant soil-water content, which could be adjusted by moving the bottom of the glass tube up (wetter soil) or down (drier soil) relative to the top of the foam stack. The graduated cylinders were used to estimate the mass of water lost from each pot due to transpiration after correcting for evaporation using control systems that did not include plants.

Growth experiment

Prosopis velutina seedlings were grown in six growth chambers (Environmental Growth Chambers model GC15, Chagrin Falls, Ohio, USA) in a factorial design with two levels of CO_2 (38 and 76 Pa, $n=3$ chambers) and two levels of water availability (high and low, $n=6$ per chamber). The chambers were programmed to simulate average environmental conditions in Tucson, Arizona, during August when *P. velutina* germinates in response to heavy monsoon rains. A 14.5/9.5 h light/dark, 36/22°C, and 50/60% RH diurnal cycle was used. Light intensity was stepped while temperature and relative humidity were ramped linearly over the first and last 3 h of the light period. Maximum quantum flux was approximately 700 μmol m^{-2} s^{-1}, achieved by adjusting the height of the lights in each chamber. Carbon dioxide levels inside the chambers were controlled using a Li-Cor 6262 infrared gas analyzer (Li-Cor, Lincoln, Neb., USA) giving 12-min averages of ±0.05–0.07 Pa CO_2 around the set points. The Li-Cor 6262 was autocalibrated against standard gases twice daily.

For each chamber, five *P. velutina* seeds were planted in each of 20 watering systems. Seeds were obtained commercially from Granite Seed, Lehi, Utah and scarified before planting by placing them in boiling water for 7 s. The potting medium was a naturally occurring sandy loam from a nearby ephemeral stream bed. This medium was

Fig. 1 Schematic of the self-regulating watering system used to maintain constant soil-water content. Water in the graduated cylinder (reservoir) was conducted to the soil in the growth tube (open ended pot covered on the bottom with 25 μm nylon mesh) via the stack of hygroscopic foam blocks contained in the large PVC tube. The water content of the soil was controlled by changing the height of the bottom of the air inlet tube in the reservoir relative to the top of the foam stack. Raising the bottom of this tube resulted in wetter soil while lowering it resulted in drier soil
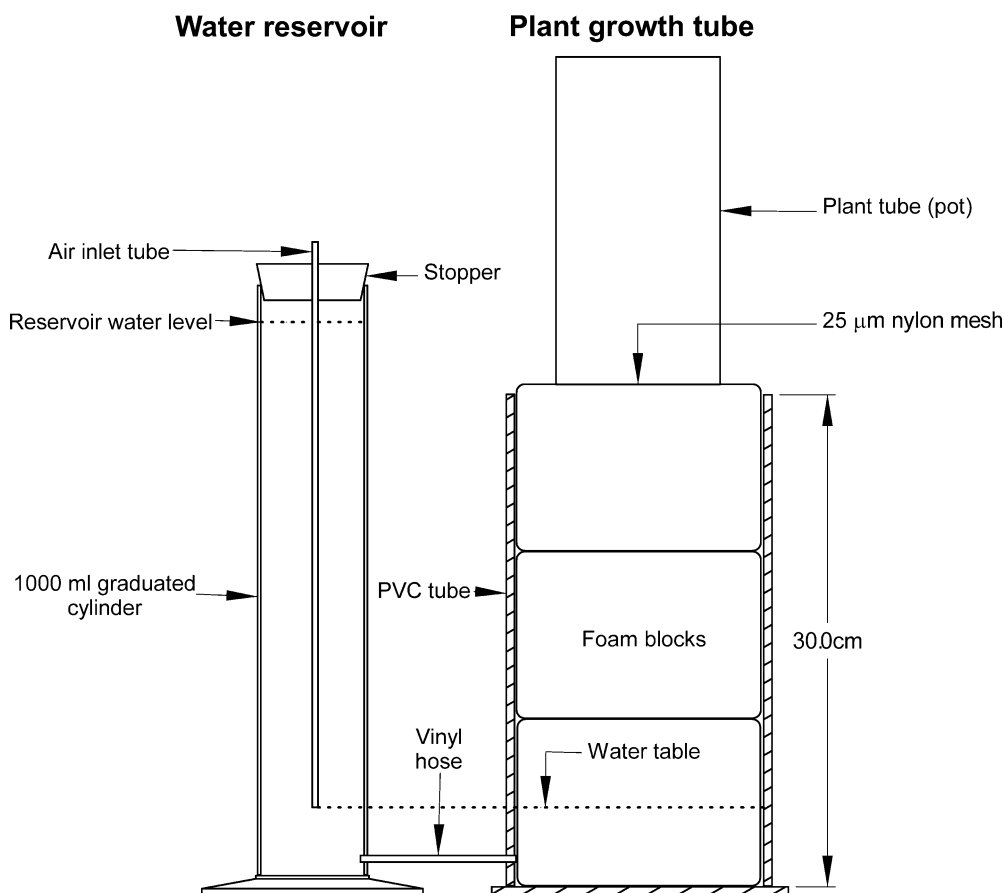
chosen because *P. velutina* seedlings germinate and grow in it naturally, and it allowed easy extraction of the root systems. Cotyledons were visible within 30 h of planting and there was close to 100% germination. At the time of planting, all watering systems were being run in the 'high' water mode by placing a 20.0 cm high stand under each graduated cylinder so that the bottom of the glass tube in each cylinder was 3.0 cm below the top of the foam stack. This resulted in a volumetric soil-water content of 14.4 ±0.4%, measured with a Delta-T ML2X Theta probe (Delta-T Devices, Cambridge, UK).

Three days after planting, eight watering systems in each chamber were chosen at random to be the evaporation controls and all seedlings were removed from them. These systems will be referred to as the "control systems" and the remainder will be referred to as the "experimental systems". For the experimental systems, two seedlings in each pot were chosen at random to be the experimental plants, and all other seedlings were removed carefully for initial measurements. One week after planting, half of the control systems and half of the experimental systems in each chamber were chosen at random to be the low water treatment. The stands were removed from under the graduated cylinders in these systems, lowering the bottom of the glass tube to approximately 23.0 cm below the top of the foam stack to give a volumetric soil water content of 4.9±0.4%. These values for the high and low water treatments are similar to those found in the sandy-loam substrate of ephemeral streams around Tucson during the monsoon. The volume of water lost from each graduated cylinder was recorded every 2 days at which time the cylinders were refilled. The mass of water transpired from each pot at the start and end of the experiment was estimated by subtracting the average mass of water lost due to evaporation in the appropriate controls from the mass of water lost from the appropriate experimental systems.

The experiment was terminated 6 weeks after planting. For both the initial and final harvests, leaf areas were estimated using a Li-Cor LI3000 leaf area meter and root areas were estimated using a flatbed scanner and image analysis software (WinRhizo, Regent Instruments Inc., Quebec, Canada). Dry mass of leaves, stems, and roots was determined after drying for 7 days at 80°C. Dried leaf material was ground and homogenized before analysis for total N using a PerkinElmer 2400 Series II elemental analyzer (PerkinElmer Analytical Instruments, Shelton, USA). All values for plant mass, leaf area, and water use were divided by 2 to give an average value for each plant in each pot. Time-averaged values for the growth analysis variables were calculated using t=39 days, which was the interval between the initial and the final harvests.

Analysis

The models show there is a strong likelihood that several growth analysis variables will be negatively correlated. Negative correlations can cause a range of statistical problems, one of which is a reduction in the power to detect true differences among treatment groups when using univariate analysis of variance (ANOVAs) on each variable separately. To minimize these problems and to provide deeper insight into the relationships among the growth analysis variables, multivariate analysis of variance (MANOVA) was used to test the main effects of CO_2, water availability, and their interaction on hierarchical groupings of the growth analysis variables. MANOVA maximizes the power to detect differences among groups when dependent variables are correlated while also controlling the experiment-wise error rate at a nominal level (Bray and Maxwell 1985).

The experiment was based on a cross-nested three-factor mixed effects design. The fixed factors were CO_2 concentration and water availability which were fully orthogonal to each other. Chamber was a random factor and nested in the CO_2 treatment. The general ANOVA model used was $y_{ijkm} = \mu + CO_{2i} + water_j + chamber_{k(i)} + (CO_2 \times water)_{ij} + (water \times chamber)_{jk(i)} + e_{m(ijk)}$. The CO_2 main effect was tested using the chamber mean square as the error term while the main effect of water availability and the $CO_2 \times$ water interaction were tested using the water $\times$ chamber interaction mean square. The water $\times$ chamber interaction was not significant in any analysis.

A combination of univariate ANOVA and discriminant function analysis was then used to identify which dependent variables contributed most to group separation for effects that were significant after MANOVA (Spector 1977). If the $CO_2 \times$ water interaction term in the MANOVA was significant, discriminant function analysis was performed using all four treatment groups. If the interaction term was not significant, groupings corresponding to significant main effects were used. For brevity, results from the univariate ANOVAs and the discriminant function analyses are not shown.

All growth analysis variables except the CRGR were transformed using natural logarithms. This not only improved multivariate normality and homogeneity of variances but also simplified interpretation of the results by making the growth analysis models additive instead of multiplicative. All variables conformed adequately with the assumptions of MANOVA and ANOVA.

Results

Whole plant and component relative growth rates

Univariate ANOVA revealed a marginal increase in the RGR in response to increasing water availability ($F_{(1,4)}$=5.79, P=0.07, Fig. 2a); however, the main effect of CO_2 and the CO_2 by water interaction were both nonsignificant ($F_{(1,4)}$=0.78, P=0.43, and $F_{(1,4)}$=0.01, P=0.93 respectively). Nevertheless, partitioning the RGR into CRGR showed highly significant multivariate main effects for both CO_2 and water availability, but their interaction was not significant (Table 2, Fig. 2b–d).
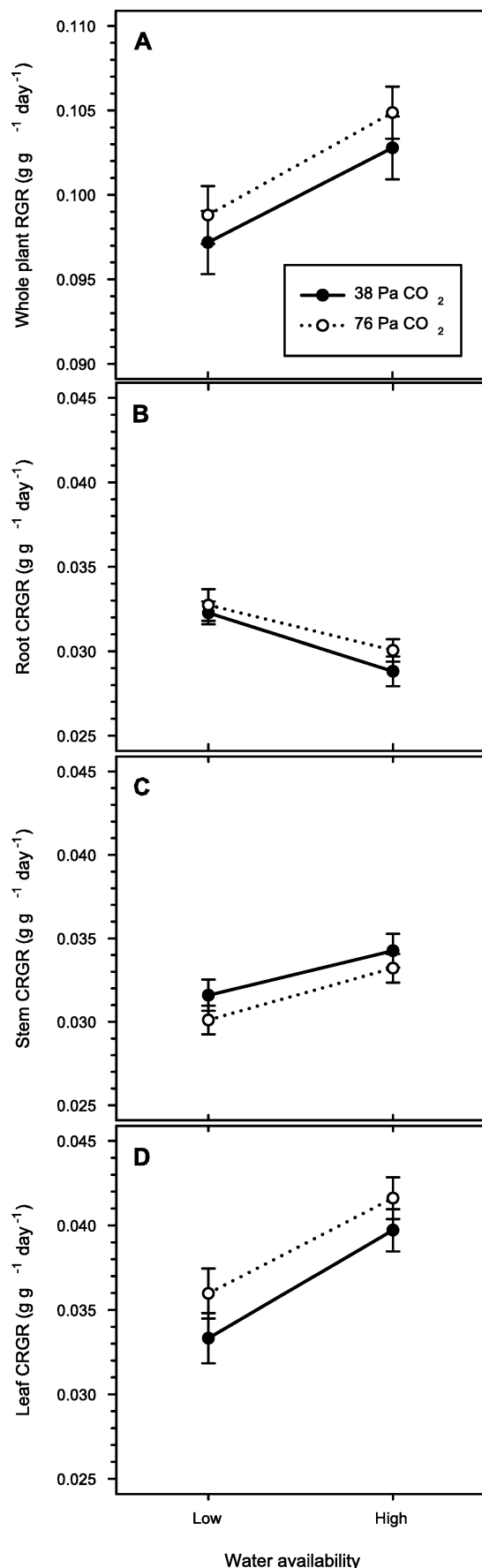
Fig. 2a–d Interaction plots showing the mean (±1 SE) effect of CO_2 and water availability on: **a** whole-plant RGR, **b** root CRGR, **c** stem CRGR, **d** leaf CRGR. Note that adding the values in panels *B*, *C*, and *D*=whole-plant RGR (panel *A*)

Table 2 MANOVA results for hierarchical groupings of the growth analysis variables

Effect	Wilk's Λ	F	df	P
Root, stem and leaf component RGR				
CO_2	0.035	36.640	3, 4	0.0023
Water availability	0.028	114.484	3, 10	<0.0001
CO_2 × water	0.793	0.869	3, 10	0.4889
ULR and LAR				
CO_2	0.343	4.80	2, 5	0.0687
Water availability	0.359	9.83	2, 11	0.0036
CO_2 × water	0.927	0.43	2, 11	0.6609
RGR_{LA}, LAR, and ΔLMA				
CO_2	0.276	3.501	3, 4	0.1288
Water availability	0.020	166.630	3, 10	<0.0001
CO_2 × water	0.600	2.227	3, 10	0.1478
SLA and LMR				
CO_2	0.8660	0.39	2, 5	0.6978
Water availability	0.0434	121.31	2, 11	<0.0001
CO_2 × water	0.8495	0.97	2, 11	0.4077
N_{prod} and N_{area}				
CO_2	0.094	24.06	2, 5	0.0027
Water availability	0.498	5.55	2, 11	0.0215
CO_2 × water	0.849	0.98	2, 11	0.4062
E_{prod} and E				
CO_2	0.0074	335.39	2, 5	<0.0001
Water availability	0.5867	3.88	2, 11	0.0532
CO_2 × water	0.4533	6.63	2, 11	0.0129
E_{prod}, WURA and S_R:S_L				
CO_2	0.0047	282.71	3, 4	<0.0000
Water availability	0.1276	22.78	3, 10	0.0001
CO_2 × water	0.2550	9.74	3, 10	0.0026

The leaf and root CRGRs increased with increasing CO_2 while the stem CRGR decreased. The ANOVA and discriminant analysis results for this effect gave conflicting rankings for the contribution of the stem and leaf CRGRs to the separation of the CO_2 treatment groups, suggesting that correlations between these two variables influenced their multivariate response to CO_2. However, the range of values for both the univariate *F* ratios and the discriminant function coefficients were narrow, suggesting that all three CRGRs contributed to separation of the CO_2 treatment groups, with the root CRGR apparently contributing the least. Overall, the increases in the leaf and root CRGRs in response to elevated CO_2 were offset somewhat by the reduction in the stem CRGR, resulting in a nonsignificant net effect of CO_2 on whole plant RGR.

The response of the component RGRs to the water treatment was consistent with general allocation patterns of plants subjected to increasing water availability in that the root CRGR decreased while the leaf CRGR increased (Fig. 2b,d). There was also an increase in the stem CRGR

with increasing water availability (Fig. 2c), however this was balanced by the reduction in the root CRGR. The univariate F ratios and standardized coefficients from the discriminant analysis showed that the leaf CRGR was the variable responsible for the greatest contribution to the separation of the water treatment groups, followed by the root and then the stem CRGRs. Thus, the marginal increase in whole plant RGR in response to increasing water availability was due primarily to an increase in the leaf CRGR.

Whole-plant final biomass displayed a similar response to RGR with a marginal increase from 0.66±0.03 (1 SE) to 0.83±0.04 g with increasing water availability ($F_{(1,4)}$=5.90, P=0.07), while both the interaction term and CO_2 main effect were nonsignificant ($F_{(1,4)}$=0.09, P=0.78, and $F_{(1,4)}$=4.28, P=0.11 respectively).

Unit leaf rate and leaf area ratio

Partitioning the RGR into the ULR (physiological index) and the LAR (morphological index) revealed a significant effect of water availability, a marginal effect of CO_2, and no interaction (Table 2, Fig. 3a, b). For the main effect of water, both the univariate F ratios and the discriminant analysis confirmed that the ULR contributed most to group separation. However, examination of Fig. 3a shows that the ULR increased by only a small amount in response to increasing water availability, and that the LAR (Fig. 3b) was effectively unchanged. Thus, the marginal increase in whole plant RGR with increasing water availability was due to this small increase in the ULR. A similar pattern was observed for the contributions of the ULR and LAR to the separation of the CO_2 treatment groups, although the

Fig. 3a–f Interaction plots showing the mean (±1 SE) effect of CO_2 and water availability on: **a** ln ULR, **b** ln LAR, **c** ln RGR_{LA}, **d** ln ΔLMA, **e** ln SLA, **f** ln LMR. Panels E + F=B. Note that the range of values used for the Y-axes (1.5) in this figure is the same as for Fig. 4
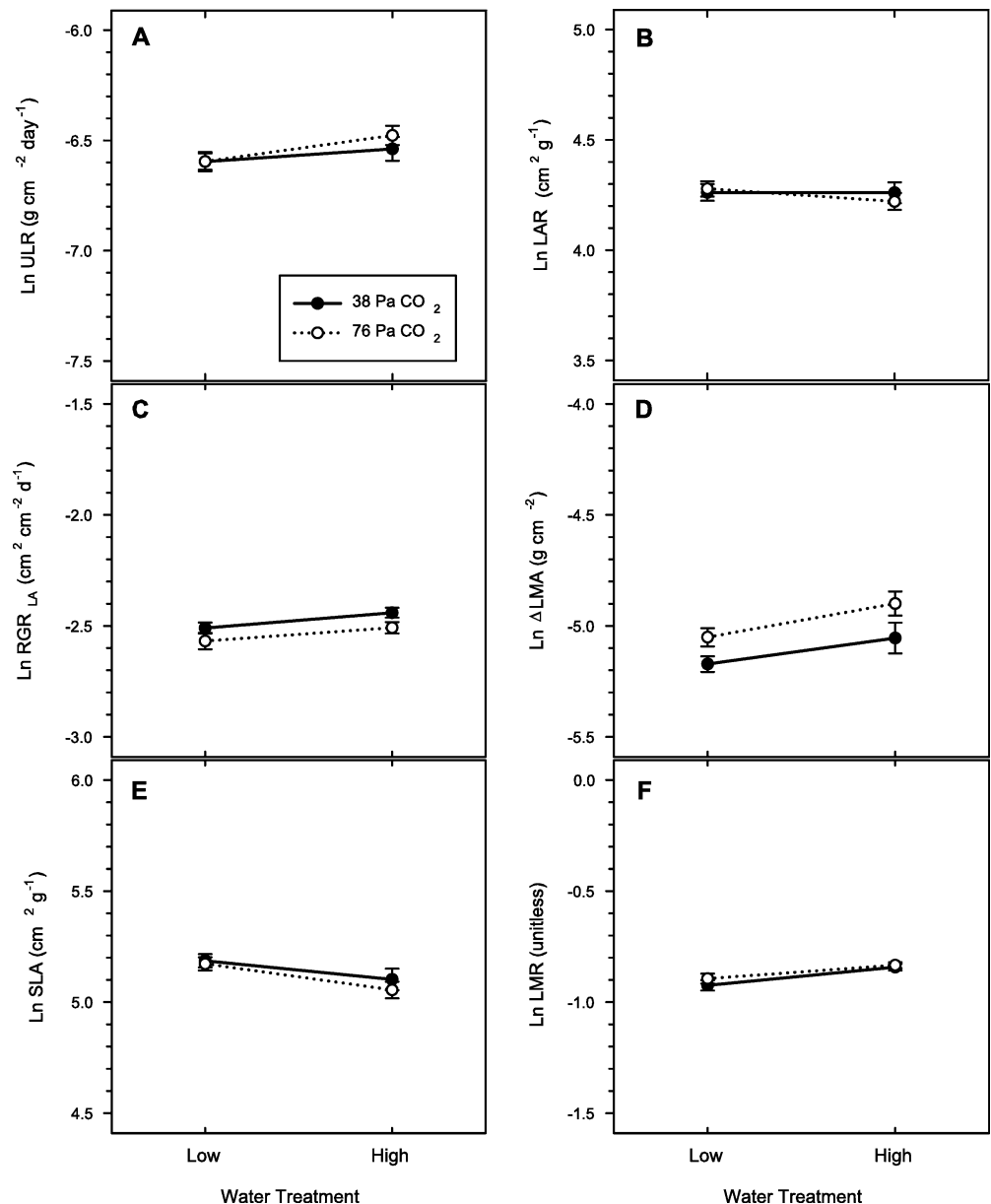
small magnitude of this effect suggests it was not biologically important.

Leaf growth

Partitioning the leaf CRGR into the leaf-area RGR, the LAR, and the change in leaf mass relative to leaf area (ΔLMA) revealed a highly significant main effect of water availability, but the CO_2 main effect and the CO_2 by water interaction were both nonsignificant (Table 2). As mentioned above, the LAR did not show any clear response to the treatments, but both the RGR_{LA} and ΔLMA increased from low to high water availability (Fig. 3c, d). However, the univariate F ratios and discriminant function coefficients provided conflicting rankings regarding the relative importance of each variable

to group separation. This suggests that correlations among these variables affected their multivariate response to increasing water availability, and therefore a conservative interpretation that both variables played some role in group separation seems appropriate. Thus, the increase in leaf CRGR with increasing water availability appears to be due to both an increase in the growth rate of leaf area and an increase in the thickness or density of the leaf tissue.

Partitioning the LAR into the SLA and LMR also revealed a highly significant effect of water availability, but again the CO_2 main effect and the interaction term were both nonsignificant (Table 2). The SLA decreased and the LMR increased with increasing water availability (Fig. 3e, f). The univariate F ratios and discriminant function coefficients confirmed that the LMR contributed more to group separation than the SLA; however, the negatively correlated responses of these two variables

Fig. 4a–f Interaction plots showing the mean (± 1 SE) effect of CO_2 and water availability on: **a** ln N_{prod}, **b** ln N_{area}, **c** ln E_{prod}, **d** ln E, **e** ln WURA, **f** ln $S_R:S_L$. The range of values used for the Y-axes (1.5) in this figure is the same as for Fig. 3. Note that panels A+B, C+D, and C+E +F all=ln ULR (Fig. 3a), and panels E+F=D
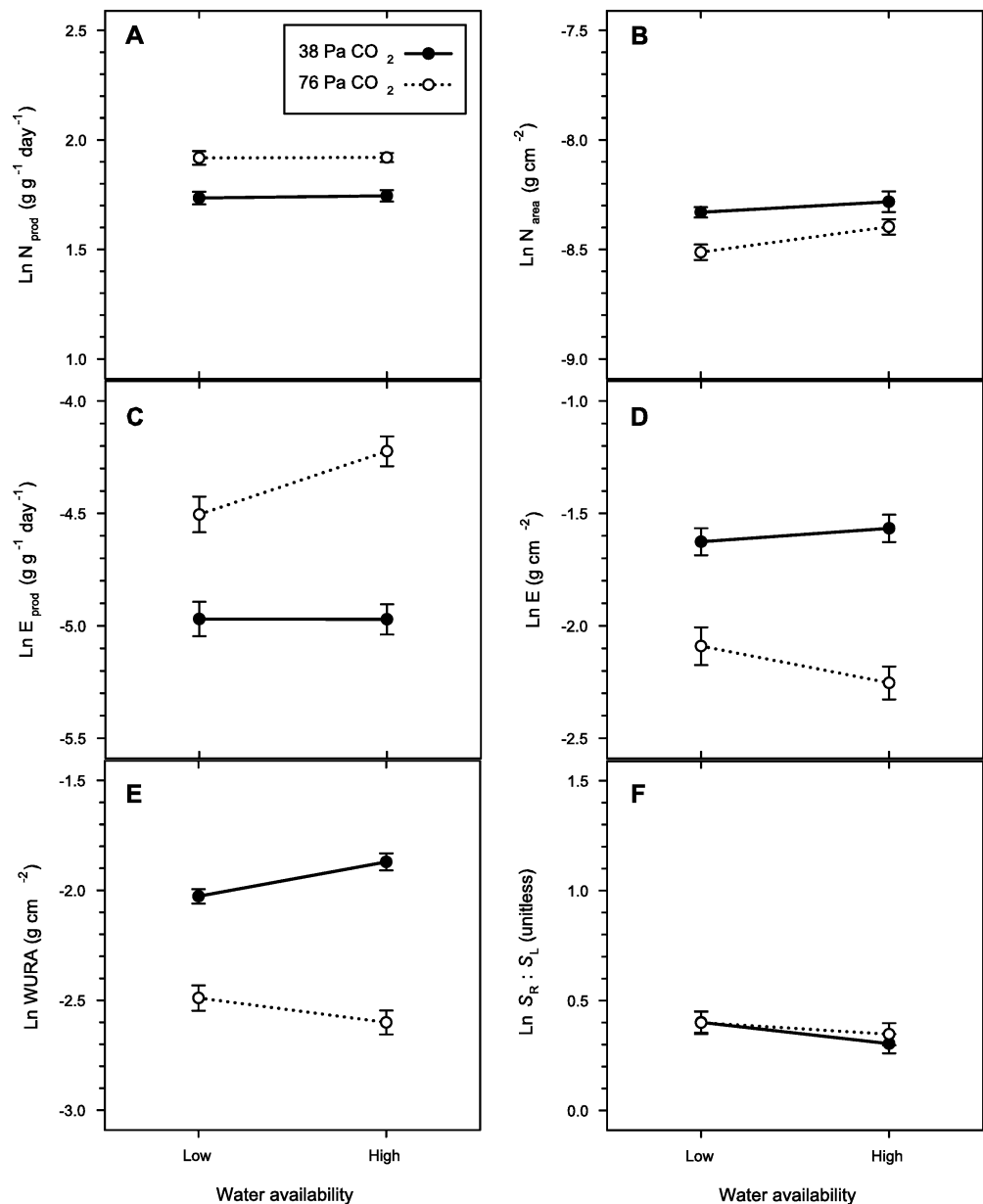
cancelled each other out, resulting in no net change in the LAR in response to a change in water availability. When considered in the context of Eq. 2, these responses to increasing water availability indicate that there was a proportional increase in leaf area with whole-plant mass from low to high water availability, but that leaf mass increased disproportionately more than whole-plant mass. These results for the SLA and LMR are in agreement with those for the RGR_{LA} and ΔLMA.

Leaf nitrogen productivity and N_{area}

Partitioning the ULR into N_{prod} and N_{area} revealed significant main effects of CO_2 and water, but the interaction term was not significant (Table 2). Nitrogen productivity increased and N_{area} decreased from low to high CO_2 partial pressure (Fig. 4a, b). The univariate F ratios and discriminant analysis confirmed that the increase in nitrogen productivity was responsible for most of the separation between the CO_2 treatment groups, although the opposing trends of N_{prod} and N_{area} cancelled each other out, resulting in no net effect of CO_2 on the ULR. There was no effect of elevated CO_2 on the total mass of leaf N, but leaf-N concentration was significantly reduced ($F_{(1,4)}=195.55$, $P<0.001$) in association with the increase in leaf CRGR (discussed above). These results show that the reduction in N_{area} with elevated CO_2 was caused by the dilution of leaf N. Data for whole-plant N were not available so we cannot say how elevated CO_2 may have affected N uptake/fixation or the relative allocation of whole-plant N to leaves.

Nitrogen productivity was not affected by water availability, but there was a small increase in the average N_{area} at high compared to low water availability (Fig. 4b). The ANOVA and discriminant analysis confirmed that the significant multivariate response to increasing water availability was driven by the increase in N_{area}. Thus, the marginal increase in the RGR in response to increasing water availability was due to an increase in N_{area}. Increasing water availability increased the total mass of N in leaves ($F_{(1,4)}=30.58$, $P=0.005$) without affecting leaf-N concentration.

Transpiration productivity and transpiration

Partitioning the ULR into E_{prod} and E revealed a highly significant main effect of CO_2, a significant interaction term, and a marginally nonsignificant main effect of water availability (Table 2). Examination of Fig. 4c, d illustrates the nature of the significant multivariate interaction term. At ambient CO_2, E_{prod} did not appear to change with increasing water availability. Elevated CO_2 increased the average E_{prod}, but this increase was greater at high compared to low water availability. The response of E, on the other hand, was opposite that of E_{prod}. While there was little effect of water availability on E at ambient CO_2, elevated CO_2 caused an overall reduction in E and this

reduction was greater at high, compared to low, water availability. It was these opposing trends in E_{prod} and E at elevated CO_2 that drove the significant interaction term in the MANOVA. However, these responses of E_{prod} and E to elevated CO_2 cancelled each other out, resulting in no net effect of CO_2 on the ULR. The small increase in the ULR with increasing water availability was due primarily to the relatively high E_{prod} of the group exposed to high CO_2 and high water availability.

The univariate F ratios for the interaction term and the standardized coefficients for the first root of the discriminant function corresponding to the interaction term gave conflicting rankings for the contribution of transpiration productivity and transpiration to the separation of the four treatment groups. This conflict indicates that the correlation between these two variables was important in their overall multivariate response. However, in this situation there is no definitive answer to the question of which variable contributed most to group separation. In this case, because the absolute range of the values for the univariate F ratios and the discriminant function coefficients was narrow, and the range of actual responses on the natural log scale was comparable for both variables (Fig. 4c, d), a conservative interpretation that both variables were important for group separation seems appropriate.

Transpiration productivity, water uptake per unit root area, and root area to leaf area ratio

Partitioning the ULR into E_{prod}, WURA, and S_R: S_L revealed highly significant multivariate responses to the main effects of CO_2 and water availability in addition to a significant interaction term (Table 2, Fig. 4c, e, f). Focusing on the interaction term, the univariate F ratios and discriminant analysis confirmed that the WURA drove the separation of the four treatment groups, followed to a lesser degree by E_{prod}. The S_R:S_L did not appear to contribute to group separation. In general, the response of the WURA was similar to that for E, which was not surprising considering the small response of the S_R:S_L to the treatments (Fig. 4f). Because of this, these results will not be discussed further.

Linking leaf nitrogen productivity and transpiration productivity: the ratio of transpiration to N_{area}

The response of the ratio E/N_{area} to the treatments was qualitatively similar to the response of E. There was a significant interactive effect of CO_2 and water availability ($F_{(1,4)}=20.35$, $P=0.011$, Fig. 5a) which was driven by the relatively low response of the group subjected to high CO_2 and high water availability. The response of this group was also largely responsible for the significant main effects of CO_2 ($F_{(1,4)}=36.60$, $P=0.002$) and water availability ($F_{(1,4)}=17.28$, $P=0.014$). Averaged over water treatments, elevated CO_2 increased E_{prod} and N_{prod}, but reduced E/

N_{area}, E, and N_{area}. Since there was no statistical effect of CO_2 on the RGR of the plants, dW in Eq. 8 did not change with CO_2 and thus the increases observed in E_{prod} and N_{prod} with elevated CO_2 were related solely to the reductions in E and N_{area}. The reduction in the ratio E/N_{area} with increasing CO_2 showed that the reduction in E was proportionately greater than the reduction in N_{area}, and because dW was not affected by CO_2, E_{prod} increased proportionately more than N_{prod}. This response to elevated CO_2 was most pronounced for the high water availability group, which had a higher N_{area} and lower E than the low water availability group in this CO_2 treatment.

Whole-plant water use

Univariate ANOVA on the volume of water used per plant over the last 2 days of the experiment revealed a highly significant interactive effect of CO_2 and water availability ($F_{(1,4)}=28.31$, $P=0.006$, Fig. 5b). Increasing the availability of water led to greater water consumption per plant at ambient CO_2 but not at elevated CO_2. When averaged over the water treatments, elevated CO_2 caused a highly

Fig. 5a, b Interaction plots showing the mean (±1 SE) effect of CO_2 and water availability on: **a** the natural log of the ratio E/N_{area}, and **b** whole-plant water use over the last 2 days of the experiment

significant reduction in whole-plant water use of approximately 41% ($F_{(1,4)}=709.0$, $P<0.0001$, Fig. 5).

Discussion

Despite only a marginal increase in the growth of *P. velutina* seedlings in response to increasing water availability, the multivariate analysis of the extensions to classical growth analysis used here, in conjunction with the subsoil watering system, revealed highly significant direct effects of increasing atmospheric CO_2 and water availability on physiological and morphological variables. Elevated CO_2 resulted in the reallocation of growth from the stems to the roots and leaves in a manner that was independent of water availability. While the data suggested that the combined increase in leaf and root growth was greater than the reduction in stem growth, this was not sufficient to cause a statistically significant increase in whole-plant growth. Even though the literature indicates that increased growth is a general response of plants to elevated CO_2, it is not ubiquitous, as demonstrated by the quantitative reviews of Poorter et al. (1996) and Curtis and Wang (1998). It is also worth mentioning here that most of the corresponding terms in the univariate ANOVAs were nonsignificant, as were the effects of CO_2 on simplistic measures of biomass allocation such as the root:shoot ratio (results not shown). This reinforces the need for multivariate approaches to studies of plant growth, particularly when variables are likely to be negatively correlated as they are in growth analysis.

Despite the absence of an effect of CO_2 on whole-plant growth, elevated CO_2 did have large and significant effects on the physiology of the plants. Biomass production per unit leaf N and per unit water transpired increased, but N per unit leaf area and transpiration per unit leaf area both decreased. These opposing responses canceled each other out, resulting in no net effect of CO_2 on the ability of the leaves to assimilate new biomass (ULR). Increases in these indices of productivity, or analogs of them, are common responses of C_3 plants exposed to increasing atmospheric CO_2 (Eamus 1991; Field et al. 1992; Drake et al. 1997; Hsiao and Jackson 1999; Wullschleger et al. 2002), and have been reported for the related species *P. glandulosa* grown at atmospheric CO_2 ranging from approximately 20–34 Pa (Polley et al. 1994). Using the meta-analytic approach, Curtis (1996) identified a nonsignificant change in leaf N per unit area across multiple independent experiments, with positive, negative, and zero responses all being common. Similarly, Curtis (1996) and Curtis and Wang (1998) identified no statistically significant effect of CO_2 on stomatal conductance (and therefore, by inference, on transpiration per unit leaf area) across multiple independent experiments, but in both studies the mean response was negative. This emphasizes a diversity of stomatal responses to elevated CO_2, with negative responses being somewhat more common than positive ones. Polley et al. (1994) did not observe any changes in N
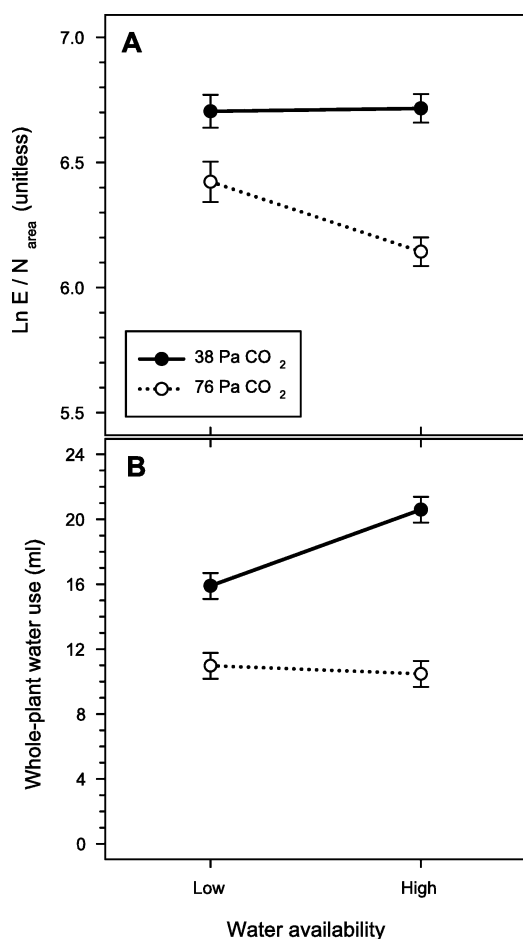
per unit leaf area or transpiration per unit leaf area for *P. glandulosa* grown at CO_2 levels ranging from 20 to 34 Pa.

Poorter et al. (1996) identified a significant increase of 22% in the average ULR across 63 C_3 species exposed to elevated CO_2, and Atkin et al. (1999) found significant increases in the ULR of 10 Australian *Acacia* species averaging 30%. The results of these authors suggest that the absence of an effect of CO_2 on the ULR of the plants in the current experiment may be unusual. The growth analytic method used by these authors and those used in the current experiment integrate net biomass gain over time but do not explicitly account for carbon losses due to respiration. The absence of a CO_2 effect on the ULR of *P. velutina* could have been due to increased respiratory carbon losses at high CO_2, but this seems unlikely because dark respiration is typically reduced substantially in plants exposed to elevated CO_2 (Curtis 1996; Drake et al. 1997; Curtis and Wang 1998). Instead, it appears that the absence of a CO_2 effect on the ULR of *P. velutina* was due to a combination of biochemical and substrate-supply limitations.

A reduction in leaf N per unit area implies a reduction in carboxylation capacity per unit leaf area because the majority of leaf N is found in the proteins of the Calvin cycle (including Rubisco) and in the thylakoid membranes (Evans 1989). In addition, under the controlled environmental conditions used in the current experiment, changes in transpiration were likely dominated by changes in stomatal conductance as opposed to changes in the diffusional gradient for water vapor between the leaf and air. Thus, elevated CO_2 would have also caused a reduction in leaf conductance to CO_2 and the supply of substrate to the sites of carboxylation. However, doubling atmospheric CO_2 partial pressure would have increased the diffusional gradient for CO_2 into the leaf, and this appears to have compensated for both the reduction in stomatal conductance and the reduction in carboxylation capacity associated with a lower leaf N. The net effect of doubling CO_2 was a significant increase in the leaf N productivity and the transpiration productivity, but no change in the overall capacity to produce new biomass per unit leaf area.

Increasing water availability also resulted in the reallocation of growth, this time from the roots to the leaves and stems, with the increase in leaf growth being sufficient to cause a marginally significant increase in whole-plant growth. The increased leaf growth was realized as an increase in both the leaf area and the thickness or density of the leaf tissue (i.e., an increase in leaf mass per unit area); however, the increase in leaf area was proportional to the increase in whole-plant mass. The increase in leaf mass per unit leaf area resulted in more N per unit leaf area and therefore a higher capacity to assimilate new biomass per unit leaf area (ULR). In terms of the water economy of the plants, the increase in leaf growth was due largely to the substantially higher transpiration productivity of the group subjected to high CO_2 and high water availability. This group had the lowest transpiration and therefore potentially the lowest conduc-

tance to CO_2, but it also had the highest leaf N of the two groups in the high CO_2 treatment. Thus, compared to the low water availability group in this CO_2 treatment, the increase in carboxylation capacity experienced by the high water availability group due to its higher leaf N appears to have over-compensated for it having the lowest conductance. The net effect was an increase in the capacity to assimilate new biomass per unit of water transpired and per unit leaf area.

We observed a highly significant reduction of approximately 41% in the volume of water transpired by *P. velutina* during the last 2 days of the experiment in response to a doubling of CO_2. This large reduction in whole-plant water use occurred because the reduction in transpiration per unit leaf area at elevated CO_2 was not offset by a proportional increase in total leaf area. Several long-term field experiments (reviewed by Drake et al. 1997; Wullschleger et al. 2002) on plant communities in which the dominant species also showed reductions in water use under elevated CO_2 demonstrated slower rates of soil moisture depletion that resulted in higher soil-water content for at least part of the growing season in plots exposed to elevated CO_2. The reduction in water use observed for *P. velutina* in the current experiment has perhaps the greatest ecological implications for this species because it suggests that under a future climate scenario, seedlings may deplete soil moisture at a slower rate than they do currently. This could facilitate seedling survival between intermittent rain events, and corroborates the conclusions of Polley et al. (1994, 1999b, 2003) that increasing levels of atmospheric CO_2 may facilitate the establishment of mesquite seedlings through a reduction in soil water depletion, particularly in situations where competition for water with grasses is reduced by commercial grazing.

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